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itle Page

Prostaglandin E Receptor Subtype EP4 Agonist Serves Better to Protect

Cochlea than Prostaglandin E1

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## 1 Introduction

2 Prostaglandin E1 (PGE1) has long been clinically used as a vasodilator, and has been  
3 proven to be effective for diverse circulatory disorders. Disorders associated with  
4 cochlear blood flow have been considered some of the principle causes of sudden  
5 sensorineural hearing loss (SSHL) [1], providing the rationale for the clinical use of  
6 several vasodilators, including PGE1, for treatment of SSHL. Although PGE1 is a  
7 therapeutic option for SSHL, its clinical benefit remains controversial [2-4]. PGE1  
8 binds primarily to E-prostanoid receptors (EP) 1–4 [5], resulting in a variety of  
9 biological effects, including vasodilation. In the central nervous system, some EP  
10 signaling pathways mediate neurotoxic effects, but others, paradoxically, appear to  
11 mediate protective effects [6]. Therefore, activation or inhibition of specific EPs might  
12 have superior therapeutic potential than does PGE1 [6-8].

13 Previous studies have focused on the roles of EP4 in the cochlea and on cochlear  
14 protection by pharmacological activation of EP4. EP4-deficient mice show slight  
15 hearing loss and are susceptible to noise-induced hearing loss [9]. Local application of  
16 an EP4 agonist has been shown to significantly attenuate noise-induced hearing loss in  
17 mice [9] and guinea pigs [10]. These findings strongly suggest the superior potential of  
18 EP4 agonists, compared with PGE1, for cochlear protection in clinic. However,

comparative assessments of the efficacy of local application of EP4 agonists and PGE1, which are crucial to precede clinical trials, have not been performed in cochlear protection investigations. Therefore, this investigation aimed to examine whether an EP4 agonist offered superior protective effects on cochleae, as compared with PGE1, against noise trauma. For this investigation, a guinea pig model of noise-induced hearing loss was used to compare the protective effects of ONO-AE1-437, an EP4 agonist, with those afforded by PGE1, following local application.

## Material and methods

### *Experimental animals*

Hartley guinea pigs, weighing 350–400g, were purchased from Japan SLC (Hamamatsu, Japan). The Animal Research Committee of the Graduate School of Medicine, Kyoto University, Japan, approved all of the experimental protocols. Animal care was supervised by the Institute of Laboratory Animals of the Graduate School of Medicine, Kyoto University. All experimental procedures involving animals were performed in accordance with the National Institutes of Health's (USA) *Guide for the Care and Use of Laboratory Animals*.

# 1    *Drug application*

2    The EP4 agonist, ONO-AE1-437, and PGE1, alprostadil (both from Ono  
3    Pharmaceutical, Osaka, Japan) were applied to the round window of guinea pig  
4    cochleae (n = 6 for ONO-AE1-437, n = 5 for PGE1), as described previously [10]. In  
5    our previous studies, ONO-AE1-329, a no-water soluble EP4 agonist was dissolved in  
6    dimethyl sulfoxide followed by dilution in physiological saline, and locally  
7    administered. As alprostadil is a water-soluble agent, a water-soluble EP4 agonist,  
8    ONO-AE1-437 was chosen for the present study. Both agents were dissolved in  
9    physiological saline to a final concentration of 1 mg/mL. Under general anesthesia with  
10    midazolam (10 mg/kg; intramuscular) and xylazine (10 mg/kg; intramuscular), the left  
11    otic bulla of experimental animals was opened to expose the round window membrane.  
12    A piece of gelatin, previously immersed in a solution of either the EP4 agonist or PGE1,  
13    was placed on the round window membrane of each animal. For the animals in the  
14    control group, a piece of gelatin immersed in physiological saline was applied (n = 5).

15

# 16    *Noise exposure and auditory brainstem response (ABR) recording*

17    Immediately after drug application, animals were exposed to 4-kHz octave band noise at  
18    120-dB sound pressure level (SPL) for 5 hours in a ventilated-sound exposure chamber

fitted with speakers driven by a noise generator and a power amplifier. A 1/2-inch condenser microphone and a fast Fourier transform analyzer (both from Sony, Tokyo, Japan) were used to monitor and calibrate sound levels at multiple locations within the chamber to ensure uniformity of the stimulus. The stimulus intensity varied by a maximum of 3-dB SPL across the measured sites within the exposure chamber. ABRs were recorded at frequencies of 4, 8, and 16 kHz before noise exposure, and on Days 3, 7, 14, and 21 after exposure. The thresholds of the ABRs at each frequency were determined, as described previously [10]. To test effects of local drug application on hearing, ABR recording was performed in normal guinea pigs (n = 4) following placement of a piece of gelatin immersed in physiological saline on the round window membrane.

### *Histological assessment*

At the conclusion of the experiment (post-exposure Day 21), each cochlea was subjected to histological analysis. Three regions of the cochlear sensory epithelia, at a distances of 30–50% (second turn), 50–70% (mid-basal portion), and 70–90% (basal portion) from the apex [11], were used for quantitative assessments of hair cell loss. Immunohistochemical staining for myosin VIIa and F-actin labeling by phalloidin was

conducted to label the inner hair cells (IHCs) and the outer hair cells (OHCs). Anti-myosin VIIa rabbit polyclonal antibody (dilution, 1:500; Proteus BioSciences, Ramona, CA, USA) was used as the primary antibody, and Alexa 568-conjugated goat anti-rabbit immunoglobulin G (dilution, 1:500) was used as the secondary antibody. After immunostaining for myosin VIIa, the specimens were stained with fluorescein-phalloidin (1:400; Molecular Probes, Eugene, OR, USA) and examined by confocal microscopy (TCS SP2; Leica Microsystems, Wetzlar, Germany). Nonspecific labeling was tested by omitting the primary antibody from the staining procedures. The numbers of IHCs and OHCs in 0.2-mm-long regions of the apical, middle, and basal portions of the cochleae were independently counted by 3 investigators; the average of the 3 counts was used in subsequent analyses.

### *Statistical analysis*

The overall effects of applied drugs on ABR threshold shifts were examined using two-way factorial analysis of variance with the post-hoc Fisher protected least significant difference test (Fisher's PLSD). Differences in the numbers of IHCs and OHCs in each region of cochleae were compared between experimental groups using one-way factorial analysis of variance with Fisher's PLSD. *P* values <0.05 were

considered statistically significant. Values were expressed as the mean and the standard deviation (SD).

## Results

### *ABR threshold shifts*

The time course of the ABR threshold shifts in noise-exposed animals at 4, 8, and 16 kHz are shown in Fig. 1. The overall effects of applied drugs were significant at 4, 8, or 16 kHz respectively ( $p < 0.0001$ ). The differences in the threshold shifts between the EP4 agonist- and PGE1-treated cochleae were shown to be significant at 4, 8 or 16 kHz ( $p < 0.0001$ ), and those between the EP4 agonist- and saline-treated cochleae were significant at 4, 8 or 16 kHz ( $p < 0.0001$  for 4 or 16 kHz,  $p = 0.0004$  for 8 kHz). No significant differences in the threshold shifts were found between the PGE1- and saline-treated cochleae at each frequency ( $p = 0.73, 0.06, 0.36$  for 4, 8, 16 kHz). No significant elevation of ABR thresholds was found in normal guinea pigs after local application of gelatin immersed in saline.

### *Histological assessment*

Immunostaining for myosin VIIa and phalloidin staining for F-actin demonstrated



severe degeneration of the OHCs in the second turn, mid-basal, and basal portions of the PGE1-treated cochleae (Fig. 2A, C, E). By contrast, OHC degeneration was limited in the EP4 agonist-treated cochleae (Fig. 2B, D, F). The IHCs were preserved in both experimental groups (Fig. 2A-F). Quantitative assessments revealed significant differences in the numbers of surviving OHCs among three groups in each portion (Fig. 3A;  $p = 0.013, 0.028, 0.038$  for the second, mid-basal, basal portion). In the second portion, significant differences in the numbers of surviving OHCs were found between the PGE1- and saline-treated cochleae ( $p = 0.039$ ) and between the EP4 agonist- and saline-treated cochleae ( $p = 0.04$ ). In the mid-basal and basal portion, the numbers of surviving OHCs in the EP4-treated cochleae were significantly higher than those in the PGE1- or saline-treated cochleae (Fig. 3A;  $p = 0.026$  or  $0.012$  for EP4 v.s. PGE1 or saline in the mid-basal,  $p = 0.021$  or  $0.028$  for EP4 v.s. PGE1 or saline in the basal). No significant differences were observed in the numbers of surviving IHCs among three groups (Fig. 3B).

## Discussion

The expression of four EP subtypes in the cochlea have been demonstrated [9, 10, 12-14], suggesting physiological or pathophysiological roles of EPs in the auditory

1 function [8]. Previous studies have indicated the involvement of EP4 in the  
2 physiopathology of cochleae and the therapeutic capability of EP4 agonists for  
3 noise-induced hearing loss [9, 10], suggesting the potential of EP4 agonists as  
4 therapeutic agents for acute sensorineural hearing loss. As PGE1 has also often been  
5 used as a therapeutic option for SSHL, the ultimate goal of this experiment was to  
6 provide preclinical evidence regarding an improved beneficial therapeutic option.  
7 Before conducting a clinical trial to examine the efficacy of EP4 agonists for the  
8 treatment of SSHL, demonstration of the differential efficacy of EP4 agonists and PGE1  
9 in an animal model of acute sensorineural hearing loss was desirable. The present  
10 results clearly demonstrated that the protective effects of an EP4 agonist on the cochleae  
11 were superior to those of PGE1, both functionally and histologically, suggesting that  
12 specific activation of EP4 can boost the therapeutic potential of PGE1 for SSHL.

13 Previously, we examined effects of the lipophilic EP4 agonist, ONO-AE1-329 on  
14 noise-induced hearing loss in a guinea pig model and demonstrated significant  
15 protection of cochleae in both pre- and post-traumatic treatment. The hearing gain  
16 following post-traumatic treatment was significant, but not as obvious as seen in  
17 pre-traumatic treatment. To clarify the difference in protective effects between a specific  
18 EP4 agonist and PGE1, pre-traumatic treatment was employed in the present study. In

the current study, a water soluble EP4 agonist, ONO-AE1-437 was investigated. Considering its potential clinical application, the water-soluble nature may be beneficial.

Both EP2 and EP4 induce intracellular production of cyclic adenosine monophosphate (cAMP), while activation of EP3 results in decreased cAMP concentrations [6-8].

Therefore, EP2 agonists or EP3 antagonists might have similar effects on noise-induced hearing loss, as did the EP4 agonist in this study, and this should be investigated in future studies. In addition, it is also necessary to examine the effects of EP4 agonists in other models of acute sensorineural hearing loss. Prior to clinical application of local EP4 agonists for treatment of acute sensorineural hearing loss, determination of the therapeutic treatment window will also need to be estimated.

In conclusion, the current study showed that local EP4 agonist treatment was superior to local PGE1 treatment for the protection of auditory function and hair cells against noise-induced damage in guinea pigs. Additional research will be required to translate the findings of this study into recommendations for regular clinical application of EP4 agonists for the treatment of SSHL.

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6 ONO-AE1-437 and PGE1.

7

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9 responsible for the content and writing of the paper.

10

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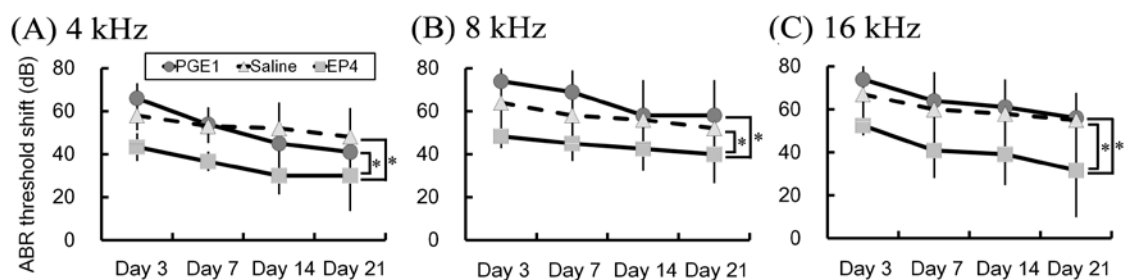
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## 1 Figure legends

2 Fig. 1: Alterations in threshold shifts of the auditory brain-stem responses (ABRs) in  
3 treated cochleae at frequencies of 4 (A), 8 (B), and 16 kHz (C). The overall effects of  
4 applied drugs were significant at 4, 8 or 16 kHz ( $p < 0.0001$ ). The differences in ABR  
5 threshold shifts at 4, 8, and 16 kHz between the E-prostanoid receptor 4 (EP4) agonist-  
6 and prostaglandin E1 (PGE1)- or saline-treated cochleae were significant (\*). Bars  
7 represent the SD.



8  
9 Fig. 2: Immunostaining for myosin VIIa (myo, red) and F-actin labeling with phalloidin  
10 (pha, green) of cochlear sensory epithelia in the second turn, mid-basal, and basal  
11 portions. Severe loss of outer hair cells (OHC) was observed in the prostaglandin  
12 E1-treated cochlea (A, C, E). Degeneration of OHCs was limited in the specimen  
13 treated with an E-prostanoid 4 receptor agonist (B, D, F). No significant difference in  
14 inner hair cells (IHC) was observed between the 2 treatments. Bars represent 25  $\mu$ m.

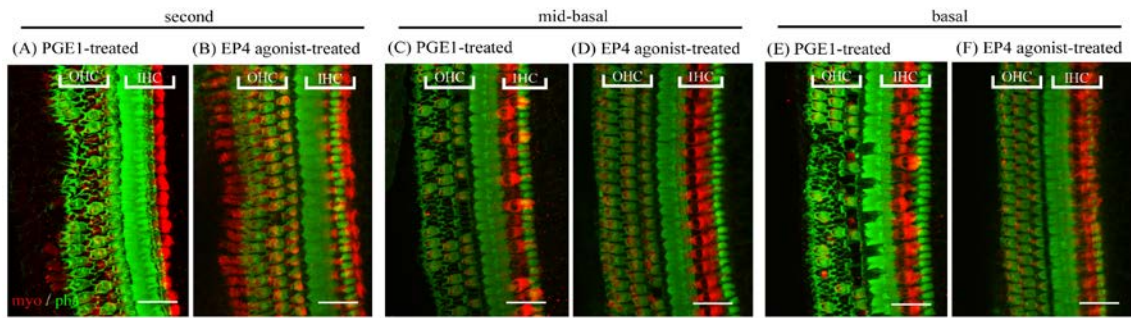


Fig. 3: Numbers of surviving outer (OHC, A) and inner hair cells (IHC, B) in the second, mid-basal, and basal portions of treated cochleae. Asterisks indicate statistical significance. Bars represent the SD.

